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Probiotic Properties of Lactic Acid Bacteria Isolated from Croatian Fresh Soft Cheese and Serbian White Pickled Cheese

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Summary

The aim of this study is to gain insight into the probiotic potential of autochthonous lactic acid bacteria (LAB) isolated from artisanal fresh soft and white pickled cheeses. Eleven out of 86 LAB isolates from traditionally produced artisanal fresh soft and white pickled cheeses which survived the most rigorous simulated gastrointestinal tract conditions and did not show resistance to antibiotics were subjected to further evaluation for functional probiotic properties. The ability of the examined strains to assimilate cholesterol in the presence of bile salts was strain dependent, with the highest percentage of cholesterol assimilated by strain *Lactobacillus brevis* BGGO7-28 possessing S-layer proteins on its cell surface. The growth of strains with mannitol or lactulose as the only carbon source was better than with fructooligosaccharides (FOS) and inulin as prebiotic substrates, which should be considered in the production of synbiotics. Moreover, the results demonstrated that the strains were highly adhesive to human enterocyte-like Caco-2 cells and to a lesser extent to HT29-MTX cells, with the exception of strain *Lb. brevis* BGGO7-28, which showed similar percentage of adhesion to both cell lines. This strain was the only one with the acidic cell surface, while other examined strains have the cell surfaces with electron donor and basic properties. In addition, all selected strains decreased the proliferation of gut-associated lymphoid tissue (GALT) cells, suggesting possible immunomodulatory potential of the isolates. Finally, the number of viable cells in dry active preparations after lyophilisation depended on the lyoprotectant used (inulin, FOS or skimmed milk), as well as on the strain subjected to lyophilisation. In conclusion, the results obtained in this study demonstrate that particular dairy LAB isolates exhibit strain-specific probiotic properties. Thus, they could be further examined as part of mixed autochthonous starter cultures for traditional cheese production under controlled conditions.

Key words: probiotics, lactic acid bacteria, Caco-2 cells, HT29-MTX cells, gut-associated lymphoid tissue (GALT), immunomodulatory activity

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Introduction

In recent years, worldwide interest in the use of lactic acid bacteria (LAB) for health promotion and disease prevention in humans and animals has significantly increased. The term 'functional starters' relates to the bacterial cultures that possess specific properties contributing to food safety and sensory, technological, nutritional or health attributes. Due to the long history of safe use in human consumption, LAB currently have the 'Qualified Presumption of Safety' (QPS) status (*1*) and some probiotic LAB strains are already consumed as part of fermented food products or as dietary supplements (*2*,*3*). According to the FAO/WHO expert consultation group, probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (*4*). Over the past years the results of research in the field of probiotics have indicated that spectrum of LAB possessing probiotic features is broadening. However, beneficial effects of probiotics are shown to be strain specific, pointing to the need to use various natural sources to identify new probiotic candidates (*5*–*7*). Therefore, collection of LAB from spontaneously fermented autochthonous products is of great significance, since artisanal products are still manufactured in a traditional way in households located at specific ecological spots and they can serve as a valuable source of microbiota containing LAB with new properties.

Western Balkan Countries region is a distinct geographical area in Europe with the tradition of manufacturing artisanal dairy products by spontaneous milk fermentation without the addition of commercial starter cultures. Due to the specific manufacturing process, these products may represent a source of distinct autochthonous strains that could have probiotic potential and serve as 'natural starter cultures' (*8*). Our previous research on autochthonous LAB strains indicated that significant genetic diversity exists amongst analysed microorganisms (*9*).

The main aim of this study is to gain an insight in the probiotic potential of LAB natural isolates and the selection of new probiotic strains. In order to identify and select new probiotic candidates with specific beneficial effects, various screening systems have been used (*7*). The guidelines proposed by the FAO/WHO (*4*) for testing the probiotic potential of a given strain *in vitro* and/or *in vivo* are based on several safety and health criteria. Therefore, the survival of LAB isolates in the conditions simulating those in the gastrointestinal (GI) tract, along with their resistance to low pH, and tolerance to pepsin, bile salts and pancreatin were regularly examined. Functional properties (adhesion ability, bile salt hydrolase activity, assimilation of cholesterol and different prebiotics) as well as technological properties (lyophilisation with different lyoprotectants) of selected autochthonous LAB isolates were examined. Besides, recent studies have shown that probiotics exhibit health beneficial effects by directly modulating or down-regulating immune system through modification of immune response in gut-associated lymphoid tissue (GALT), preventing in that way the symptoms of inflammatory bowel disease, allergies and asthma (*10*). Hence, primary GALT cells have been suggested as an improved *in vitro* model for studying the interactions of microorganisms, due to the fact that they are non-transformed and non-tumourigenic (*11*). Moreover, due to accelerated appearance of bacteria resistant to more than one antibiotic (multiple resistance), one of the goals of this study is the analysis of antibiotic susceptibilities of potential probiotic strains and provision of their safe application in practice.

Materials and Methods

Bacterial strains and growth conditions

LAB strains used in this study were isolated previously from white pickled and fresh soft cheeses traditionally manufactured in mountain regions in Serbia and lowland regions in Croatia, respectively, as described in detail by Golić *et al.* (*9*). In order to distinguish the origins of the isolates, strains were marked as BG when isolated from white pickled cheeses from Serbia and ZG when isolated from fresh soft cheeses from Croatia. Out of 86 LAB strains 45 were isolated from ten white pickled artisanal cheeses collected in Serbia from South Morava region (BGAL1, BGAL2, BGAL3 and BGLE1), Golija mountain (BGGO5, BGGO11 and BGGO7) and the mountainous region of Eastern Serbia (BGVL1, BGBU1 and BGRE2), and 41 strains were isolated from nine fresh soft artisanal cheeses collected in Croatia from Prigorje (ZGPR1, ZGPR2 and ZGPR3), Bilogorsko-Podravska region (ZGBP4, ZGBP5 and ZGBP6) and Zagorje (ZGZA7, ZGZA8 and ZGZA9).

The selected 86 LAB strains were further investigated and stored at –80 °C in an appropriate medium, M17 or De Man Rogosa Sharpe (MRS) broth (Biolife, Milano, Italy) supplemented with 30 % (by volume) of glycerol (Gram-mol, Zagreb, Croatia). Stock LAB cultures were subcultured twice in MRS broth at 30 °C (*Lactococcus* and *Leuconostoc* strains) or at 37 °C (*Lactobacillus* and *Enterococcus* strains) prior to the experiments.

Cumulative effect of simulated gastric and intestinal juices on the survival of the isolates of autochthonous LAB

The overnight culture of each isolate of autochthonous LAB was centrifuged (10 000×*g* for 1 min), washed twice and resuspended in 0.5 % (by mass per volume) of sterile NaCl solution. Washed cell suspensions were added into simulated gastric juice. The average initial viable cell count was about 8.7 log CFU/mL. Simulated gastric juice (pH=2; 3 g/L of pepsin and 0.5 % (by mass per volume) of NaCl) and intestinal juice (3 g/L of oxgall, 1 g/L of pancreatin and 0.5 % (by mass per volume) of NaCl) were prepared according to Kos *et al.* (*12*). Pepsin from porcine stomach mucus was obtained from Sigma-Aldrich (St. Louis, MO, USA), pancreatin from Fluka Biochemika (Buchs, Switzerland) and oxgall from Difco (Detroit, MI, USA). After the exposure of the washed cell suspensions to simulated gastric juice (residence time 2 h), bacterial cells were centrifuged at 10 000×*g* for 1 min and resuspended in intestinal juice (residence time 4 h). Changes in total viable cell count were determined at the beginning and the end of treatment by pour plate method using MRS agar. Plates were incubated at optimal growth conditions for 48 h.

Antibiotic susceptibility testing

The overnight culture of each autochthonous LAB isolate was centrifuged (10 000×*g* for 1 min), washed twice and finally suspended in 0.5 % (mass per volume) of NaCl to a turbidity equivalent to that of McFarland standard no. 1. Autochthonous LAB isolates were examined for susceptibility to 12 different antibiotics: penicillin G, ampicillin, bacitracin, vancomycin, erythromycin, gentamicin, clindamycin, chloramphenicol, streptomycin, neomycin, tetracycline and novobiocin by agar disc diffusion method according to Mayerhofer *et al.* (*13*), using antimicrobial susceptibility test discs (Oxoid, Basingstoke, UK). Antibiotic discs were placed on agar plates inoculated with bacterial suspensions and incubated at appropriate growth conditions for 48 h. Inhibition zones around the discs were measured and expressed in diameter size (in mm), including the disc.

For the Etest, bacterial suspensions with a turbidity equivalent to McFarland standard no. 1 were inoculated on MRS agar plates. After the surface of the plates was dried, the Etest strips (M.I.C. Evaluator™, Oxoid Ltd, Basingstoke, UK) of the tested antibiotic (ampicillin, clindamycin, gentamicin, streptomycin, tetracycline, erythromycin and vancomycin) were applied. The plates were incubated under the same conditions as the ones applied in antibiotic disk diffusion tests. The minimal inhibitory concentrations (MICs) were read directly from the test strip according to the manufacturer's instructions.

Lyophilisation

Lyophilisation of the bacterial cultures was performed according to Beganović *et al.* (*14*). Late exponential phase cells, grown in MRS medium, were collected by centrifugation at 3300×*g* for 15 min, washed twice and resuspended in 5 mL of phosphate-buffered saline (PBS) with or without the addition of lyoprotectant at 5 % (by mass per volume), including skimmed milk (Dukat, Zagreb, Croatia), inulin (Difco) and fructooligosaccharides (FOS) Raftilose® P95 (Orafti, Tienen, Belgium). Prepared cell suspensions were frozen at –80 °C for 12 h. Frozen cultures were freeze-dried in lyophilizer, model Christ Alpha 1–2 LD (B. Braun Biotech International GmbH, Melsungen, Germany). Viable cell count (CFU/mL) was determined before and after freeze-drying using the standard pour-plating method. Each experiment was conducted in triplicate.

Quantitative determination of bile salt hydrolase activity and cholesterol assimilation

Determination of bile salt hydrolase (BSH) activity was performed by quantification of the amount of cholic acid liberated from conjugated bile salt sodium taurocholate (2 mg/mL added to MRS medium) by solvent extraction method, during 24 h of incubation of the examined strains (*15*).

The modified *o*-phthalaldehyde method was used to determine the amount of cholesterol (AppliChem, Darmstadt, Germany) in the supernatant, in the washing buffer where precipitated cholesterol was redissolved, and in the cell extract after cell disruption to obtain assimilated cholesterol after 24 h of incubation in MRS containing 3 mg/mL of oxgall and 0.2 mg/mL of cholesterol (*16*).

Assimilation of prebiotic substrates

Prebiotic substrates mannitol (Difco), lactulose (Merck, Darmstadt, Germany), FOS and inulin were added to carbohydrate-free MRS broth to a final mass per volume ratio of 1 %. Prepared media were inoculated with washed cell suspension of culture grown overnight in MRS. The growth of autochthonous LAB strains after 24 h of incubation in MRS broth in which glucose was substituted with one of the prebiotic substrates was detected by absorbance measurement at 620 nm (microplate reader, model LKB 5060-006, LKB Vertriebs GmbH, Vienna, Austria), where the growth of respective strains in carbohydrate-free MRS basic medium served as control.

Properties of bacterial cell surface and adhesion of LAB strains to intestinal cell lines

Microbial adhesion to solvents (MATS) for determination of hydrophobicity, and Lewis acid/base properties of the bacterial cell surfaces of the examined strains were evaluated as described by Kos *et al.* (*17*).

The colonocyte-like cell lines Caco-2 and HT29-MTX were used to determine the adhesion ability of the LAB isolates to mucosal surfaces in the intestine. Caco-2 cell lines were purchased from the European Collection of Cell Cultures (ECACC No. 86010202, Public Health England, Porton Down, UK) and HT29-MTX cell lines were kindly supplied by Dr. T. Lesuffleur (UMR S 938, INSERM, Paris, France) (*18*). The culture and maintenance of the cell lines were carried out following standard procedures (*19*) using Advanced Dulbecco's Modified Eagle Medium (DMEM; Gibco, Invitrogen, Paisley, UK) for Caco-2 and HT29-MTX cells supplemented with heat-inactivated foetal bovine serum (5 % for Caco-2, 10 % for HT29-MTX), L-glutamine (2 mM) and with a mixture of antibiotics (10 U/mL of penicillin, 10 mg/mL of streptomycin and 50 μg/mL of gentamicin). Media and reagents were purchased from PAA Laboratories (Pasching, Austria). Intestinal cells were seeded in 24-well plates and cultivated until confluent differentiated monolayers were obtained. LAB isolates were cultured for 24 h and after washing twice with Dulbecco's PBS solution (Sigma-Aldrich), they were resuspended in the corresponding cell line media without antibiotics at a concentration of about 10^8 CFU/mL. Cellular monolayers were also carefully washed and LAB suspensions were added at a ratio of LAB/eukaryotic cells of about 10:1. Adhesion experiments were carried out for 1 h at 37 °C, 5 % CO_2 and, afterwards, wells were gently washed to release unattached bacteria before proceeding with the lysis of cellular monolayers using 0.25 % Trypsin-EDTA solution (PAA Laboratories). Dilutions of samples, before and after adhesion, were made in PBS solution and LAB counts were determined on MRS or GM17 agar plates. The adhesion was calculated as the percentage of CFU of the adhered LAB strain per CFU of the added LAB strain. Experiments were carried out in two replicated plates and in each plate two wells were used per sample.

Isolation of GALT from rat and its proliferation in the presence of non-viable LAB strains

The LAB strains were grown in the same manner as described above. Overnight LAB cultures were harvested by centrifugation, washed twice with PBS and resuspended in 5 mL of the same buffer with a final cell suspension of 10^7 CFU/mL determined by plate counting. Cells were inactivated by UV light (in a UV chamber, 15 W, CAMAG, Muttenz, Switzerland) for 3 cycles of 30 min each. Plate counting was carried out after UV treatment to corroborate the absence of live LAB strains that could recover in proper medium. UV-inactivated LAB isolates were then divided in single-use aliquots, frozen in liquid N_2 and stored at –80 °C until use (*20*).

The isolation of GALT from rat was approved by the Animal Experimentation Ethical Committee at the University of Belgrade, Serbia, and performed strictly in accordance with International Directives. Three healthy male Wistar rats (6–8 weeks old) were purchased from the Farm of the Military Medical Academy, Belgrade, Serbia. All animals were housed in the animal facility at the Faculty of Pharmacy, University of Belgrade, Serbia, under the standard conditions (23–25 °C with 12:12 h reversed light/dark cycle). Rats were fed with a commercial diet having unlimited access to standard rat food and tap water. Each animal was anaesthetized with CO_2 and, once assured the loss of corneal reflex, its intestine was excised from the jejunum to the ileocaecal junction. Small intestine was transfered in the ice-cold Hanks' balanced salt solution (HBSS, without calcium and magnesium, Gibco, Invitrogen) and kept at 4 °C until processing. Finally, the animals were euthanized using the increased CO_2 concentration.

The isolation of lymphocytes, *i.e.* Peyer´s patch lymphocytes (PPL) and intestinal epithelium lymphocytes (IEL) from GALT was carried out as described by Hidalgo-Cantabrana *et al.* (*21*). Briefly, small pieces of the cleaned small intestine were incubated with HBSS containing gentamicin 500 mg/mL (AppliChem GmbH), penicillin 20 IU/mL (PAA Laboratories), streptomycin 2 μg/mL (PAA Laboratories) and 10 mM 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid (HEPES buffer; Lonza, Walkersville, MD, USA). Treatments with HBSS containing 2 mM DTT and 10 mM HEPES, pH=7.2, and HBSS containing 1 mM EDTA and 1 mM HEPES, pH= 7.2, were applied to release the IEL lymphocyte subset. Incubation in RPMI-1640 medium (Sigma-Aldrich) with 2 mM L-glutamine (PAA Laboratories), 25 mM HEPES, 10 % heat-inactivated FBS, 100 μg/mL of each streptomycin (Sigma-Aldrich) and ampicillin (Sigma-Aldrich), and collagenase 100 IU/mL (Gibco, Invitrogen) was used to isolate PPL subset of lymphocytes. The PPL and IEL present in the supernatants were purified using Percoll gradient (Sigma-Aldrich) (from 66 to 47 and then to 25 %), and then resuspended in RPMI-1640 medium with antibiotics.

To quantify the response of GALT to different tested factors, $2{\cdot}10^5$ lymphocyte cells were incubated with UV-inactivated LAB at a ratio of 1:5 in RPMI-1640 medium containing antibiotics at 37 °C with 5 % CO_2 for 4 days. GALT cells were grown in triplicate in 96-well round-bottom microtiter plates. The proliferation of GALT lymphocytes was determined by Amersham Cell Proliferation Biotrak ELISA System (GE Healthcare, Buckinghamshire, UK) following the manufacturer´s instructions. Results were compared with negative control (lymphocytes growing in complete RPMI-1640 medium with antibiotics) to test the immunomodulating capability of each factor.

Statistical analysis

The results are presented as the average values of three batches for each of the samples with error bars representing the standard deviations. Analysis of variance (ANOVA) was used to determine statistically significant differences among treatments using STATISTICA v. 9.0 software (StatSoft Inc., Tulsa, OK, USA). *Post hoc* Duncan's multiple range test was used as a guide for pair comparisons of the treatment means. The differences between treatments that are described subsequently as being significant were determined at $p<0.05$.

Results and Discussion

Selection of potential probiotic strains

In a previous study, 86 LAB isolates with different profiles obtained by rep-PCR using $(GTG)_5$ fingerprinting were identified by 16S rDNA sequencing (*9*). Later analysis of BGGO7-28 strain by amplified fragment length polymorphism (AFLP™) method provided by Belgian Coordinated Collections of Microorganisms/ Laboratory for Microbiology (BCCM/LMG, University of Ghent, Ghent, Belgium) identification service revealed that this strain is actually *Lactobacillus brevis*.

The ability to survive transit through the GI tract in high viable cell counts is an important probiotic property, hence 86 strains were tested for their tolerance to these most rigorous conditions. Selection of potential probiotic strains which could survive in the GI tract was performed on the basis of the measurements of strain survival in simulated gastric (pH=2) and intestinal juices. From the set of 86 LAB strains, isolated and identified in the previous study (*9*) from ten white pickled (marked as BG) and nine fresh soft cheeses (marked as ZG), 60 strains did not survive after 2 h of exposure to simulated gastric juice and 4 h of exposure to simulated intestinal juice. Among 26 LAB strains that survived in simulated GI tract conditions, strains *Lactococcus lactis* ssp. *lactis* BGLE1-6, *Lactobacillus plantarum* ZGPR2-25, *Lb. plantarum* ZGPR3-18, as well as *Lc. lactis* ssp. *lactis* biovar. *diacetylactis* BGAL1-1, *Lb. brevis* BGGO7-28, *Ec. faecium* ZGPR1-54 and *Ln. pseudomesenteroides* ZGBP4-14 achieved the best survival, as shown in Table 1. Although strains *Lb. casei* BGGO5-7 and *Lb. rhamnosus* BGGO5-47 did not survive the rigorous GI tract conditions, they were considered for further examination since they could be of interest as functional starter cultures in cheese production as the only representatives of their species. Besides, the rigorous GI tract conditions could be overcome by microencapsulation of the bacterial cells in different matrices. Recently, several authors have reported that the delivery of microencapsulated bacteria in food carriers improves their survival

Table 1. Survival of autochthonous LAB strains exposed to simulated gastrointestinal (GI) tract conditions and after lyophilisation with skimmed milk, inulin and fructooligosaccharides (FOS) as lyoprotectants

Strain	Cell mortality/(Δlog CFU/mL)			
	Simulated GI tract conditions*	After lyophilisation with lyoprotectants		
		Skimmed milk	Inulin	FOS
Ec. faecium ZGPR1-54	$(2.4\pm0.1)^{c}$	$(1.6\pm0.1)^{b}$	$(1.64\pm0.03)^{b}$	$(1.2\pm0.1)^{a}$
Lb. brevis BGGO7-28	$(2.3\pm0.1)^{c}$	$(0.12\pm0.01)^{a}$	$(0.58\pm0.05)^{c}$	$(0.41\pm0.04)^{b}$
Lb. casei BGGO5-7	$(8.7\pm0.1)^{h}$	$(0.02\pm0.03)^{a}$	$(0.53\pm0.07)^{b}$	$(0.8\pm0.1)^{c}$
Lb. plantarum BGGO5-3	$(4.19\pm0.03)^{f}$	$(0.7\pm0.1)^{b}$	$(0.1\pm0.2)^{a}$	$(2.74\pm0.04)^{c}$
Lb. plantarum ZGPR2-25	$(1.94\pm0.05)^{b}$	$(0.76\pm0.02)^{b}$	$(0.50\pm0.07)^{a}$	$(1.9\pm0.1)^{c}$
Lb. plantarum ZGPR3-18	$(1.88\pm0.05)^{b}$	$(1.65\pm0.04)^{c}$	$(0.1\pm0.1)^{a}$	$(0.74\pm0.08)^{b}$
Lb. rhamnosus BGGO5-47	$(8.8\pm0.1)^{h}$	$(0.30\pm0.06)^{a}$	$(0.75\pm0.04)^{b}$	$(3.6\pm0.1)^{c}$
Lc. lactis ssp. *lactis* biovar. *diacetylactis* BGAL1-1	$(2.75\pm0.07)^{d}$	$(1.03\pm0.08)^{c}$	$(0.27\pm0.02)^{b}$	$(0.2\pm0.06)^{a}$
Lc. lactis ssp. *lactis* BGLE1-6	$(1.38\pm0.04)^{a}$	$(0.7\pm0.1)^{b}$	$(0.2\pm0.2)^{a}$	$(0.88\pm0.06)^{b}$
Lc. lactis ssp. *lactis* ZGBP5-9	$(5.4\pm0.3)^{g}$	$(2.13\pm0.04)^{b}$	$(2.7\pm0.1)^{c}$	$(1.8\pm0.1)^{a}$
Ln. pseudomesenteroides ZGBP4-14	$(3.20\pm0.05)^{e}$	$(1.8\pm0.1)^{b}$	$(1.14\pm0.06)^{a}$	$(2.44\pm0.05)^{c}$

*direct transit from simulated gastric juice (pH=2, t=2 h) to simulated intestinal juice (0.3 % bile salts, t=4 h)
Values in the same column (for results obtained in simulated GI tract conditions) or row (for results obtained after lyophilisation) having different letters in superscript differ significantly ($p<0.05$)
ZG=LAB strains from soft cheese, BG=LAB strains from white pickled cheese

through the GI tract due to the buffering and protective effects of the food components (*22,23*). Moreover, strains *Lc. lactis* ZGBP5-9 and *Lb. plantarum* BGGO5-3 were selected for further examination due to their ability to survive GI tract conditions *in vitro* in the highest number among the same species isolated from traditional fresh and white pickled cheeses, respectively. Finally, all together 11 LAB strains were subjected to further evaluation as potential probiotic strains.

Antibiotic susceptibility and resistance

An important issue during selection and evaluation of new probiotics is their safety. Therefore, characterisation of the antibiotic resistance patterns of the putative probiotic strains is obligatory in order to avoid uncontrolled spreading of the antibiotic-resistance genes among bacterial species through gene transfer (*24*). The antibiotic susceptibility of eleven LAB strains that survived in simulated GI tract conditions was examined by agar disc diffusion method to 12 different antibiotics (penicillin G, ampicillin, bacitracin, vancomycin, erythromycin, gentamicin, clindamycin, chloramphenicol, streptomycin, neomycin, tetracycline and novobiocin) (data not shown). The examined *Lactobacillus* strains together with *Ln. pseudomesenteroides* ZGBP4-14 displayed phenotypic resistance to vancomycin. This is natural intrinsic resistance to glycopeptides due to the replacement of C-terminal D-alanine by D-lactate or D-serine, which results in a decreased binding of glycopeptides (*25*).

The susceptibility of 11 selected potential probiotic strains to antibiotics was additionally checked according to LAB resistance criteria proposed for antibiotics of human and veterinary importance by the European Food Safety Authority (EFSA) (*1*). MICs of ampicillin, clindamycin, gentamicin, streptomycin, tetracycline, erythromycin and vancomycin determined for each potential probiotic strain were the same or lower than the breakpoints proposed by EFSA for categorising bacteria, therefore 11 selected LAB strains could be considered as susceptible to the examined antibiotics (Table 2). Consequently, these strains were further investigated for potential probiotic properties.

Lyophilisation of LAB strains with lyoprotectants

During processing and storage, probiotic cells are exposed to a number of stress factors such as extremely high or low temperatures, low pH, high osmotic pressure and high oxygen levels (*26*). In order to exert the probiotic activity in the human intestinal tract, probiotic preparations need to contain 10^6–10^8 CFU per g of the product. Therefore, lyophilisation of the wet cell biomass was performed with skimmed milk, inulin and fructooligosaccharides (FOS) as lyoprotectants in order to obtain high viable cell counts in active dry preparations. Among 11 lyophilized LAB strains, the highest viable cell numbers of five strains: *Lc. lactis* ssp. *lactis* BGLE1-6, *Lb. plantarum* BGGO5-3, *Lb. plantarum* ZGPR2-25, *Lb. plantarum* ZGPR3-18 and *Ln. pseudomesenteroides* ZGBP4-14 were reached when inulin was used as lyoprotectant; of three strains: *Lc. lactis* ssp. *lactis* biovar. *diacetylactis* BGAL1-1, *Ec. faecium* ZGPR1-54 and *Lc. lactis* ssp. *lactis* ZGBP5-9 when FOS was used, and of another three strains: *Lb. brevis* BGGO7-28, *Lb. casei* BGGO5-7 and *Lb. rhamnosus* BGGO5-47 when skimmed milk was used as lyoprotectant (Table 1). The addition of lyoprotectants is important for bacterial cell membrane stabilisation and prevention of the irreversible damage of the cell biopolymers within the cell. Phospholipids in the cytoplasmic membrane are hydrated, and dehydration causes an increase of the van der Waals forces between carbohydrate chains,

Table 2. Minimum inhibitory concentrations (MICs) of different antibiotics determined for 11 autochthonous LAB strains

Strain	MICs/(μg/mL)						
	Amp	DA	CN	Te	S	E	Va
Ec. faecium ZGPR1-54	0.5	0.5	32	0.03	128	2	0.25
Lb. brevis BGGO7-28	0.15	0.03	2	4	64	0.06	n.r.
Lb. casei BGGO5-7	0.015	0.06	4	1	64	0.06	n.r.
Lb. plantarum BGGO5-3	0.06	0.06	8	2	n.r.	0.25	n.r.
Lb. plantarum ZGPR2-25	0.25	1	8	4	n.r.	1	n.r.
Lb. plantarum ZGPR3-18	0.12	1	8	8	n.r.	1	n.r.
Lb. rhamnosus BGGO5-47	0.25	0.02	8	8	32	0.25	n.r.
Lc. lactis ssp. *lactis* biovar. *diacetylactis* BGAL1-1	0.06	3	8	4	32	0.06	0.25
Lc. lactis ssp. *lactis* BGLE1-6	0.12	2	2	4	32	0.25	0.25
Lc. lactis ssp. *lactis* ZGBP5-9	0.06	0.015	2	4	32	0.06	0.25
Ln. pseudomesenteroides ZGBP4-14	0.03	0.25	4	8	64	0.12	n.r.

n.r.=not required according to EFSA (*1*); Amp=ampicillin, DA=clindamycin, CN=gentamicin, Te=tetracycline, S=streptomycin, E=erythromycin, Va=vancomycin; ZG=LAB strains from soft cheese, BG=LAB strains from white pickled cheese

which results in cell membrane damage. According to the previous studies, the cryoprotective effect of sugar could be attributed to the formation of hydrogen bonds between phosphate groups in phospholipids and hydroxyl groups of sugars, which results in the membrane stabilisation (*27*,*28*). When polysaccharides are applied as lyoprotectants, interaction between polysaccharides and phospholipids greatly depends on the flexibility of the structures. For example, a fructose-based polysaccharide, such as inulin, which tends to form a random coil structure, has a more profound interaction with the membrane than levan, which tends to form a helix (*29*).

Bile salt hydrolase activity and cholesterol assimilation with LAB strains

Relatively high degree of survival in the presence of bile salts in simulated small intestinal juice indicates resistance of some chosen strains to bile salts, which could be ascribed to BSH activity. BSH catalyses the deconjugation of bile salts. Free (deconjugated) bile salts have lower solubility at low pH (because deconjugation increases their p*K* values) and precipitate as a result of the fermentative metabolism of LAB (*30*). To date, BSH activity has not yet been detected in *Lactococcus* and *Leuconostoc* strains. Therefore, in this study potential activity of this enzyme was examined in chosen *Lactobacillus* and *Enterococcus* strains (Table 3). Determination of BSH activity was performed by quantification of the amount of cholic acid liberated from conjugated bile salt taurocholate added to MRS medium during 24 h of incubation of the examined strains. All strains showed low BSH activity since only 10 % and even less of taurocholate added to the growth medium was deconjugated. Simultaneously, strains were able to grow in the presence of high bile salt concentration, which indicated that some other detoxification mechanisms were included in the protection of cells from damage caused by bile salts. Namely, bile salts are detergent-like biological compounds which induce cell membrane damage, and the antimicrobial activity of bile salts against Gram-positive bacteria is the result of dissipation of the proton-motive force. It is shown that various multidrug resistance proteins (LmrCD or CmbT) could be responsible for the increased resistance of lactococci to bile salts and that active exclusion of bile salts from cells could also play a role in the bile salt adaptation phenomenon (*31*,*32*). However, it is noteworthy to emphasize that BSH activity could be a mechanism responsible for the resistance of probiotic strains to bile salts, but high activity of this enzyme in probiotic strains is not desirable. Namely, deconjugated bile salts derived from conjugated bile salts are the substrates for steroid-7α-dehydroxylase enzyme, produced by some members of intestinal microbiota in the intestinal tract. Secondary bile salts, such as deoxycholate and lithocholate, resulting from deconjugated bile salt dehydroxylation, act as promoters of carcinogenic processes in the intestinal tract (*30*). Therefore, the examined strains with the obtained low BSH activity are suitable for application as probiotics.

Table 3. Growth and bile salt hydrolase activity of autochthonous LAB strains after 24 h of cultivation at 37 °C in MRS broth supplemented with 2 mg/mL of TCA

Strain	$A_{620\ nm}$	$w(TCA_{deconjugated})$/%
Ec. faecium ZGPR1-54	$(0.53\pm0.06)^{c}$	$(8.2\pm0.6)^{b}$
Lb. brevis BGGO7-28	$(0.28\pm0.04)^{d}$	$(8.5\pm1.2)^{b}$
Lb. casei BGGO5-7	$(0.74\pm0.02)^{a}$	$(10.4\pm0.4)^{a}$
Lb. plantarum BGGO5-3	$(0.27\pm0.06)^{d}$	$(10.4\pm0.4)^{a}$
Lb. plantarum ZGPR2-25	$(0.68\pm0.05)^{b}$	$(7.8\pm0.3)^{b}$
Lb. plantarum ZGPR3-18	$(0.68\pm0.05)^{b}$	$(7.0\pm0.9)^{bc}$
Lb. rhamnosus BGGO5-47	$(0.55\pm0.03)^{c}$	$(10.4\pm2.3)^{ab}$

Values in the same column having different letters in superscript differ significantly ($p<0.05$)
TCA=sodium salt of taurocholic acid; ZG=LAB strains from soft cheese, BG=LAB strains from white pickled cheese

Moreover, already 1 % reduction in serum cholesterol is associated with an estimated reduction of 2–3 % of the risk of coronary artery disease incidence (*33*). BSH activity of LAB can decrease cholesterol solubility and reduce its uptake from the intestine, so the ability of LAB isolates for cholesterol uptake or assimilation of and co-precipitation with bile salts was also tested. The amount of cholesterol was determined in the supernatant, in the washing buffer where the precipitated cholesterol was redissolved, and in the cell extract after cell disruption to determine its assimilation by the examined strains after 24 h of incubation in MRS containing bile salts and cholesterol (Fig. 1). Fractions of cholesterol in the growth medium after 24 h of incubation were decreased for about 20 % with all tested strains. About 10 % of cholesterol precipitated under acidic conditions, which is in correlation with the level of deconjugation of taurocholic acid. Namely, cholesterol precipitation is a result of decreased solubility of deconjugated bile salts (*30*). However, 1.27 to 24.38 % of cholesterol was recovered after disruption of cells, which indicates that cholesterol was assimilated by the examined strains. Interestingly, the best cholesterol assimilation (24.38 %) was obtained by strain *Lb. brevis* BGGO7-28, which possesses S-layer proteins on its cell surface (*34*). The possible role of S-layer proteins in cholesterol assimilation remains to be elucidated as well as the possible correlation between BSH activity and cholesterol assimilation of all tested strains.

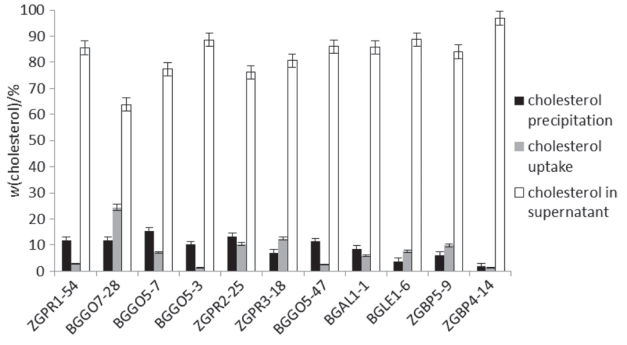

Fig. 1. Percentage of cholesterol measured in supernatants, washing buffers (precipitated cholesterol) and cell extracts (assimilated cholesterol) of autochthonous LAB strains after 24 h of cultivation at 37 °C in MRS broth supplemented with 3 mg/mL of oxgall and 0.2 mg/mL of cholesterol. ZG=LAB strains from soft cheese, BG=LAB strains from white pickled cheese

Prebiotic assimilation with LAB strains

The growth of 11 potential probiotic strains with different prebiotics as the only carbon source in MRS medium during 24 h of incubation was investigated (Fig. 2). The best growth of strains was achieved with mannitol as prebiotic substrate and with lactulose, with the exception of two strains: *Lc. lactis* BGAL1-1 and *Lb. rhamnosus* BGGO5-47. Interestingly, white pickled cheese isolates showed better growth on mannitol, while fresh soft cheese isolates grew better on lactulose. These data could be useful if synbiotic products, *i.e.* combination of probiotics and prebiotics, are prepared.

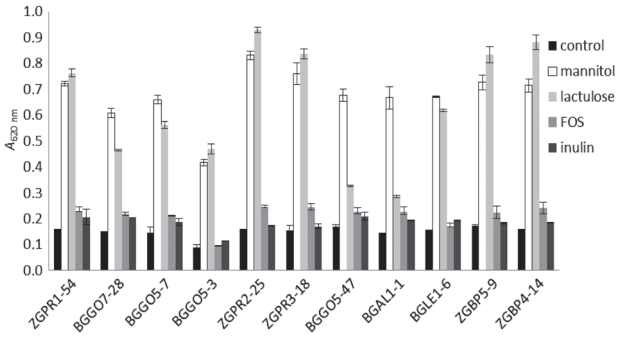

Fig. 2. Growth of autochthonous LAB strains after 24 h of incubation in MRS broth supplemented with different prebiotics instead of glucose. Control=MRS broth without glucose and prebiotics. ZG=LAB strains from soft cheese, BG=LAB strains from white pickled cheese

Adhesion ability of LAB strains

Physicochemical characteristics of the cell surface of chosen autochthonous strains play an important role in their initial interaction, which then enables specific interactions between adhesins on the bacterial cell surfaces and complementary receptors on intestinal epithelial cells (*17*).

In order to examine cell surface properties of chosen autochthonous isolates, their hydrophobicity/hydrophilicity was compared. These strains showed low to moderate hydrophobicity according to the affinity to hexane as a nonpolar solvent (Table 4). Furthermore, all tested strains revealed high affinity to chloroform, which confirmed the prevalent present basic character of LAB cell surfaces, with an exception of strain *Lb. brevis* BGGO7-28, which showed affinity to ethyl acetate, implying the acidic character of its cell surface. It should be emphasised that only this strain out of 86 tested LAB strains expresses S-layer proteins on its cell surface (*34*).

The influence of bacterial surface cell properties on the strain potential to adhere to intestinal mucosal surface was further examined. Namely, the colonisation of intestinal mucosa is an important criterion proposed by FAO/WHO guidelines for selection of strains with probiotic potential, since their health-promoting effects might be partly dependent on the persistence in the intestine and adhesion to mucosal surfaces (*35*). Using widely accepted *in vitro* models, Caco-2 and HT29-MTX cells, 11 tested strains showed higher percentage of adhesion to the Caco-2 cell line, while the adhesion ability to the HT29-MTX cell line was significantly lower ($p<0.05$). Possibly, the presence of the glycoprotein (mucin) layer in the HT29-MTX cell line could hinder the availability of cell receptors for LAB isolates. In particular, the highest percentages of adhesion to the Caco-2 cell line were exhibited by the strains *Lb. plantarum* BGGO5-3, *Ec. faecium* ZGPR1-54, *Lc. lactis* ssp. *lactis* ZGBP5-9 and *Lc. lactis* ssp. *lactis* biovar. *diacetylactis* BGAL1-1, while the highest percentages of adhesion to the HT29-MTX cell line were exhibited by the strains *Lb. plantarum* ZGPR3-18, *Lc. lactis* ssp. *lactis* BGLE1-6 and *Ln. pseudomesenteroides* ZGBP4-14 (Fig. 3). Interestingly, *Lb. brevis* BGGO7-28 showed similar percentage of adhesion to both cell lines.

Table 4. Adhesion ability of autochthonous LAB strains obtained by microbial adhesion to solvents (MATS) method

Strain	MATS/%		
	Hexane	Ethyl acetate	Chloroform
Ec. faecium ZGPR1-54	$(0.8\pm0.4)^{g}$	$(6.8\pm0.5)^{e}$	$(53.3\pm2.4)^{c}$
Lb. brevis BGGO7-28	$(6.8\pm1.0)^{e}$	$(28.4\pm1.5)^{b}$	$(18.8\pm3.1)^{e}$
Lb. casei BGGO5-7	$(19.6\pm0.4)^{c}$	$(2.8\pm0.5)^{g}$	$(65.0\pm3.2)^{a}$
Lb. plantarum BGGO5-3	$(2.4\pm0.4)^{f}$	$(12.0\pm1.1)^{d}$	$(44.7\pm1.8)^{d}$
Lb. plantarum ZGPR2-25	$(1.2\pm0.3)^{g}$	$(4.7\pm0.6)^{f}$	$(51.5\pm1.7)^{c}$
Lb. plantarum ZGPR3-18	$(3.4\pm0.7)^{f}$	$(3.3\pm0.8)^{fg}$	$(45.9\pm2.5)^{d}$
Lb. rhamnosus BGGO5-47	$(17.0\pm2.3)^{c}$	$(6.4\pm0.2)^{e}$	$(51.6\pm3.2)^{c}$
Lc. lactis ssp. *lactis* biovar. *diacetylactis* BGAL1-1	$(36.1\pm1.1)^{b}$	$(31.3\pm3.8)^{b}$	$(59.5\pm2.1)^{b}$
Lc. lactis ssp. *lactis* BGLE1-6	$(13.0\pm1.2)^{d}$	$(22.4\pm2.6)^{c}$	$(48.6\pm2.4)^{cd}$
Lc. lactis ssp. *lactis* ZGBP5-9	$(7.2\pm0.5)^{e}$	$(2.2\pm0.1)^{g}$	$(42.5\pm1.0)^{d}$
Ln. pseudomesenteroides ZGBP4-14	$(47.4\pm3.0)^{a}$	$(40.2\pm2.6)^{a}$	$(63.6\pm1.4)^{a}$

Values in the same column having different letters in superscript differ significantly ($p<0.05$)
ZG=LAB strains from soft cheese, BG=LAB strains from white pickled cheese

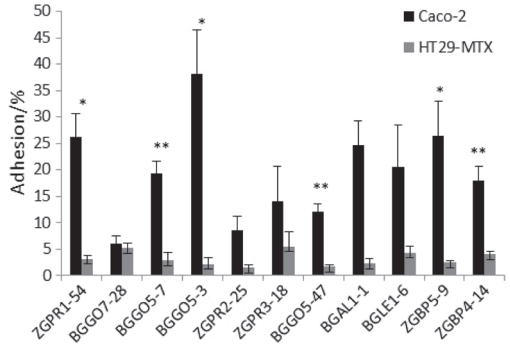

Fig. 3. Adhesion of LAB strains to Caco-2 (dark grey bars) and to HT29-MTX (light grey bars) intestinal cell lines. Bars represent significant differences between Caco-2 and HT29-MTX adhesion for each strain at: *p<0.05 and **p<0.005. ZG=LAB strains from soft cheese, BG=LAB strains from white pickled cheese

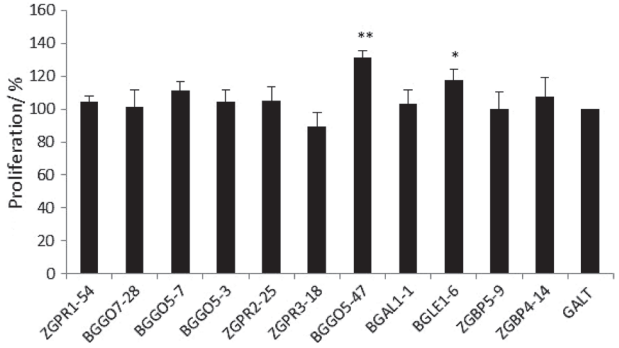

Fig. 4. Proliferation of gut-associated lymphoid tissue (GALT) cells in the presence of UV-inactivated LAB strains. Bars represent significant differences between each strain and GALT cultures without LAB at: *p<0.05 and **p<0.005. ZG=LAB strains from soft cheese, BG=LAB strains from white pickled cheese

Proliferation of GALT in the presence of UV-inactivated LAB strains

Another important characteristic of potential probiotic candidates is the capacity to modulate the immune response of the host. In general, two main effects of probiotics on host immunity are strengthening the immunological barrier through the development of the innate and adaptive immune system, or decreasing the immune responsiveness to unbalanced inflammatory conditions. Nevertheless, it is important to emphasize that the ability for immune modulation is strain- and dose-dependent, as well as the fact that well established probiotics are not effective for all human populations (*36–39*). Hence, the capability of 11 LAB strains, inactivated by UV-radiation, to elicit immune response was tested on GALT isolated from three male rats. Unstimulated cells were used as a negative control (as 100 % of proliferation). The proliferation indices of GALT measured in the presence of the stimuli are shown in Fig. 4. Specifically, the strains *Lb. rhamnosus* BGGO5-47 ($p<0.005$) and BGLE1-6 ($p<0.05$) showed the ability to increase the proliferation of GALT cells significantly, indicating the proinflammatory potential, while the strain *Lb. plantarum* ZGPR3-18 reduced the number of GALT cells in comparison with non-treated GALT cells.

According to the obtained results, among 11 autochthonous LAB isolates originating from the artisanal cheeses, the strains with the ability to elicit different immune response patterns on rat GALT are: *Lb. plantarum* ZGPR3-18, potentially involved in immunosuppressive and immune regulatory functions, and *Lb. rhamnosus* BGGO5-47 and BGLE1-6 with proinflammatory potential, suggesting the potential use of these strains in different functional foods, for example as part of mixed autochthonous starter cultures for traditional cheese production under controlled conditions. While the strain *Lb. plantarum* ZGPR3-18 could be included in the food designed for the diet of patients suffering from autoimmune disorders associated with an increased inflammatory status (allergy or inflammatory bowel disease), *Lb. rhamnosus* BGGO5-47 could have specific probiotic applications for human consumption in order to strengthen the immune system.

Conclusions

Putting all the results together, it could be concluded that the obtained *in vitro* results, although they cannot be directly extrapolated, indicate that some of the natural LAB isolates analysed in this study could have specific probiotic potential. Still, according to the FAO/WHO criteria and EFSA recommendations, it is essential to highlight that the safety and health-promoting efficacy of such functional foods need to be demonstrated in human clinical trials.

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References

1. European Food Safety Authority, Guidance on the assessment of bacterial susceptibility to antimicrobials of human or veterinary importance, *EFSA J. 10* (2012) 1–10.
2. J. Beganović, A. Leboš Pavunc, K. Gjuračić, M. Špoljarec, J. Šušković, B. Kos, Improved sauerkraut production with probiotic strain *Lactobacillus plantarum* L4 and *Leuconostoc mesenteroides* LMG 7954, *J. Food Sci. 76* (2011) 124–129.
3. A. Leboš Pavunc, J. Beganović, B. Kos, K. Uroić, M. Blažić, J. Šušković, Characterization and application of autochthonous starter cultures for fresh cheese production, *Food Technol. Biotechnol. 50* (2012) 141–151.
4. FAO/WHO, Probiotics in food. Health and nutritional properties and guidelines for evaluation, *FAO Food Nutr. Pap. 85* (2006).
5. B. Kos, J. Beganović, L. Jurašić, M. Švađumović, A. Leboš Pavunc, K. Habjanič, J. Šušković, Coculture-inducible bacteriocin biosynthesis of different probiotic strains by dairy starter culture *Lactococcus lactis*, *Mljekarstvo, 61* (2011) 273–282.
6. J. Beganović, B. Kos, A. Leboš Pavunc, K. Uroić, M. Jokić, J. Šušković, Traditionally produced sauerkraut as source of autochthonous functional starter cultures, *Microbiol. Res. 169* (2014) 623–632.
7. L. Solieri, A. Bianchi, G. Mottolese, F. Lemmetti, P. Giudici, Tailoring the probiotic potential of non-starter *Lactobacillus* strains from ripened Parmigiano Reggiano cheese by *in vitro* screening and principal component analysis, *Food Microbiol. 38* (2014) 240–249.
8. L. Topisirovic, M. Kojic, D. Fira, N. Golic, I. Strahinic, J. Lozo, Potential of lactic acid bacteria isolated from specific natural niches in food production and preservation, *Int. J. Food Microbiol. 112* (2006) 230–235.
9. N. Golić, N. Čadež, A. Terzić-Vidojević, H. Šuranská, J. Beganović, J. Lozo *et al.*, Evaluation of microbial diversity in traditional white-pickled and fresh soft cheeses from mountain regions in Serbia and low laying regions in Croatia, *Int. J. Food Microbiol. 166* (2013) 294–300.
10. A.C. Ouwehand, Antiallergic effects of probiotics, *J. Nutr. 137* (2007) 794–797.
11. G.I. Solano-Aguilar, K. Garland Vengroski, E. Beshah, J.K. Lunney, Isolation and purification of lymphocyte subsets from gut-associated lymphoid tissue in neonatal swine, *J. Immunol. Methods, 241* (2000) 185–199.
12. B. Kos, J. Šušković, J. Goreta, S. Matošić, Effect of protectors on the viability of *Lactobacillus acidophilus* M92 in simulated gastrointestinal conditions, *Food Technol. Biotechnol. 38* (2000) 121–127.
13. S. Mayerhofer, K.J. Domig, C. Mair, U. Zitz, G. Huys, W. Kneifel, Comparison of broth microdilution, Etest, and agar disk diffusion methods for antimicrobial susceptibility testing of *Lactobacillus acidophilus* group members, *Appl. Environ. Microbiol. 74* (2008) 3745–3748.
14. J. Beganović, B. Kos, A. Leboš Pavunc, K. Habjanič, M. Ogorelec, J. Šušković, Influence of freeze-drying and microencapsulation on functionality of probiotic strains in the intestinal tract, *Proceedings of the International Scientific and Professional Conference 13th Ružička Days*, Osijek, Croatia (2011) pp. 206–218.
15. J. Šušković, B. Kos, S. Matošić, V. Besendorfer, The effect of the bile salts on survival and morphology of a potential probiotic strain *Lactobacillus acidophilus* M92, *World J. Microbiol. Biotechnol. 16* (2000) 673–678.
16. B. Kos, Probiotic concept: *in vitro* investigation with chosen lactic acid bacteria, *PhD Thesis*, Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb, Croatia (2001) (in Croatian).
17. B. Kos, J. Šušković, S. Vuković, M. Šimpraga, J. Frece, S. Matošić, Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92, *J. Appl. Microbiol. 94* (2003) 981–987.
18. T. Lesuffleur, N. Porchet, J.P. Aubert, D. Swallow, J.R. Gum, J.S. Kim *et al.*, Differential expression of the human mucin genes *MUC1* to *MUC5* in relation to growth and differentiation of different mucus-secreting HT-29 cell subpopulations, *J. Cell Sci. 106* (1993) 771–783.
19. M. Nikolic, P. López, I. Strahinic, A. Suárez, M. Kojic, M. Fernández-García *et al.*, Characterisation of the exopolysaccharide 1 (EPS)-producing *Lactobacillus paraplantarum* BGCG11 and its non-EPS producing derivative strains as potential probiotics, *Int. J. Food Microbiol. 158* (2012) 155–162.
20. P. López, D.C. Monteserín, M. Gueimonde, C.G. de los Reyes-Gavilán, A. Margolles, A. Suárez, P. Ruas-Madiedo, Exopolysaccharide-producing *Bifidobacterium* strains elicit different *in vitro* responses upon interaction with human cells, *Food Res. Int. 46* (2012) 99–107.
21. C. Hidalgo-Cantabrana, M. Nikolic, P. López, A. Suárez, M. Miljkovic, M. Kojic *et al.*, Exopolysaccharide-producing *Bifidobacterium animalis* subsp. *lactis* strains and their polymers elicit different responses on immune cells from blood and gut associated lymphoid tissue, *Anaerobe, 26* (2014) 24–30.
22. R.D.C.S. Ranadheera, S.K. Baines, M.C. Adams, Importance of food in probiotic efficacy, *Food Res. Int. 43* (2010) 1–7.
23. A. Leboš Pavunc, J. Beganović, B. Kos, A. Buneta, S. Beluhan, J. Šušković, Influence of microencapsulation and transglutaminase on viability of probiotic strain *Lactobacillus helveticus* M92 and consistency of set yoghurt, *Int. J. Dairy Technol. 64* (2011) 254–261.
24. A. Leboš Pavunc, B. Kos, J. Beganović, K. Uroić, D. Bučan, J. Šušković, Antibiotic susceptibility and antimicrobial activity of autochthonous starter cultures as safety parameters for fresh cheese production, *Mljekarstvo, 63* (2013) 185–194.
25. N. Toomey, D. Bolton, S. Fanning, Characterisation and transferability of antibiotic resistance genes from lactic acid bacteria isolated from Irish pork and beef abattoirs, *Res. Microbiol. 161* (2010) 127–135.
26. A. Upadrasta, C. Stanton, C. Hill, G. Fitzgerald: Improving the Stress Tolerance of Probiotic Cultures: Recent Trends and Future Directions. In: *Stress Responses of Lactic Acid Bacteria*, E. Tsakalidou, K. Papadimitriou (Eds.), Springer, New York, NY, USA (2011) pp. 395–438.

27. S.A. Carvalho, J. Silva, P. Ho, P. Teixeira, X.F. Malcata, P. Gibbs, Relevant factors for the preparation of freeze-dried lactic acid bacteria, *Int. Dairy J. 14* (2004) 835–847.
28. X.C. Meng, C. Stanton, G.F. Fitzgerald, C. Daly, R.P. Ross, Anhydrobiotics: The challenges of drying probiotic cultures, *Food Chem. 106* (2008) 1406–1416.
29. C. Santivarangkna, B. Higl, P. Foerst, Protection mechanisms of sugar during different stages of preparation process of dried lactic acid starter cultures, *Food Microbiol. 25* (2008) 429–441.
30. M. Begley, C. Hill, C.G.M. Gahan, Bile salt hydrolase activity in probiotics, *Appl. Environ. Microbiol. 72* (2006) 1729–1738.
31. A.H. Zaidi, P.J. Bakkes, J. Lubelski, H. Agustiandari, O.P. Kuipers, A.J.M. Driessen, The ABC-Type multidrug resistance transporter LmrCD is responsible for an extrusion--based mechanism of bile acid resistance in *Lactococcus lactis*, *J. Bacteriol. 190* (2008) 7357–7366.
32. B. Filipic, N. Golic, B. Jovcic, M. Tolinacki, D.C. Bay, R.J. Turner *et al.*, The *cmbT* gene encodes a novel major facilitator multidrug resistance transporter in *Lactococcus lactis*, *Res. Microbiol. 164* (2013) 46–54.
33. J.E. Manson, H. Tosteson, P.M. Ridker, S. Satterfield, P. Herbert, G.T. O'Connor, The primary prevention of myocardial infarction, *N. Engl. J. Med. 326* (1992) 1406–1416.
34. B. Kos, K. Uroić, J. Beganović, N. Golić, N. Čadež, L. Topisirović *et al.*, Probiotic properties of autochthonous lactic acid bacteria isolated from artisanal soft cheeses, *Book of Abstracts of the 6th Central European Congress on Food, CEFood 2012*, Novi Sad, Serbia (2012) p. 498.
35. M.C. Collado, E. Isolauri, S. Salminen, Y. Sanz, The impact of probiotic on gut health, *Curr. Drug Metab. 10* (2009) 68–78.
36. M.P. Díaz-Ropero, R. Martín, S. Sierra, F. Lara-Villoslada, J.M. Rodríguez, J. Xaus, M. Olivares, Two *Lactobacillus* strains, isolated from breast milk, differently modulate the immune response, *J. Appl. Microbiol. 102* (2007) 337–343.
37. S. Van Hemert, M. Meijerink, D. Molenaar, P.A. Bron, P. de Vos, M. Kleerebezen *et al.*, Identification of *Lactobacillus plantarum* genes modulating the cytokine response of human peripheral blood mononuclear cells, *BMC Microbiol. 10* (2010) 1–13.
38. B. Evrard, S. Coudeyras, A. Dosgilbert, N. Charbonnel, J. Alamé, A. Tridon, C. Forestier, Dose-dependent immunomodulation of human dendritic cells by the probiotic *Lactobacillus rhamnosus* Lcr35, *PLoS ONE, 6* (2011) e18735.
39. S. Seifert, A. Bub, C.M. Franz, B. Watzl, Probiotic *Lactobacillus casei* Shirota supplementation does not modulate immunity in healthy men with reduced natural killer cell activity, *J. Nutr. 141* (2011) 978–984.